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TITLE: Combat-Related Heterotopic Ossification: Development of Animal Models for Identifying Mechanisms and Testing Therapeutics

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14. ABSTRACT

HO is occurring at significantly higher frequencies in the current war-wounded population than in civilian populations and it is a common and significant problematic clinical entity for war-wounded patients. The available body of evidence suggests that polytraumatic blast injuries induce HO with high frequency as a result of a combination of systemic and local factors. Prevention of HO through the development of prophylactic treatments would reduce military treatment costs and the pain and suffering of Wounded Warriors. A critical hurdle in our investigation of HO etiology, treatment, and prevention is the absence of a reliable and reproducible small animal model that can be used to characterize combat-related HO development, identify new prophylactic/therapeutic targets, and test new HO countermeasures.

15. SUBJECT TERMS

Nothing listed

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INTRODUCTION

Heterotopic ossification (HO) is the formation of mature lamellar bone within soft tissue after severe traumatic injury. Severe blast-related combat extremity injury is frequently associated with the development of ectopic bone (HO) within the soft tissue at the zone of injury and/or limb amputation during the injury repair/healing process. Recent studies have shown that heterotopic ossification (HO) is among the most common long-term reasons for re-operation in amputated limbs. The mechanism(s) involved in HO are unclear, but evidence is accumulating that HO formation is associated with specific injury patterns wherein bacterial contamination and subsequent wound colonization may be a key risk factor. Using a small animal model of blast-related extremity injury involving a combinations of combat-related injury patterns and stressors (blast, fracture, soft-tissue crush injury, limb amputation), sought to determine if (1) the presence of bioburden (Acinetobacter baumannii and methicillin-resistant Staphylococcus aureus [MRSA]) increases the magnitude of ectopic bone formation in traumatized muscle after amputation; and (2) what persistent effects bacterial contamination has on late microbial flora within the amputation site.

KEYWORDS

Heterotopic ossification, bioburden, blast overpressure, combat injury, Methicillin-resistant Staphylococcus aureus (MRSA), Acinetobacter baumannii, crush injury, amputation, inflammation, wound healing

OVERALL PROJECT SUMMARY

Task1: Bacteria inoculation dose-finding

Heterotopic ossification (HO) is the formation of mature lamellar bone within soft tissue following traumatic injury and is known to develop in approximately 65% of combat-related amputations. Symptomatic HO is characterized by persistent pain and skin ulceration disrupting prosthetic rehabilitation. Surgical excision is the only definitive management option when physical therapy and prosthetic modification fail to provide adequate relief, however it is wrought with complications. Other prophylaxis strategies, including treatment with NSAIDs or external-beam radiotherapy, are generally contraindicated in the setting of combat trauma.

Recent findings suggest that heightened and prolonged expression of inflammatory mediators may contribute to HO formation. Moreover, the combat wound is thought to provide a unique micro-environment that promotes the skewed differentiation of endogenous tissue-derived progenitor cells towards ectopic bone development within injured soft tissue. These pathways may be potentiated by bacterial colonization given

that HO is known to develop in combat-related amputations wherein early colonization with *Acinetobacter baumanni* and methicillin-resistant *Staphylococcus aureus* (MRSA) is ubiquitous. Recently, our group has developed a traumatic small animal model that incorporates a combination of critical physiologic injury patterns sustained by combat casualties including blast exposure, extremity fracture, quadricep crush injury followed by limb amputation through the zone of injur. Expanding on this rat model for the development of post-traumatic HO, we sought to evaluate the influence of wound colonization on the formation of HO.

Methods and Experimental Procedure-We exposed forty-three adult male Sprague-Dawley rats (350-400g) to 120±7 kPa blast over pressure using a shock tube followed by femur fracture, thigh crush injury and transfemoral amputation done within the zone of injury. Each wound was inoculated beneath the myodesis with 1.0 10^6 x CFUs of a highly virulent strain of either *Acinetobacter Baumannii* or *Methicillin Resistant Staphylococcus Aureus* (MRSA) which were isolated from combat wounds. A control group was injured as described above, but did not undergo inoculation. Rats were followed for visual evidence of wound infection requiring irrigation and debridement and euthanized if three serial debridements performed at least 24 hours apart did not result in clinical improvement. We performed microCT (mCT) imaging weekly for the first month and at 8 and 12 weeks post-operatively in order to measure HO volume. Samples of muscle tissue adjacent to the amputation site and bone marrow from the residual femur were harvested to determine persistence of infection. Representative animals in each treatment group underwent en bloc resection of the femur and surrounding musculature for histologic analysis.

Bacteria Culture Conditions: Acinetobacter baumannii (strain 5075) and methicillin-resistant Staphylococcus aureus (MRSA strain 107261) organisms used in this study are highly virulent, well-characterized clinical specimens isolated from combat wounds from patients treated at the Walter Reed National Military Medical Center. In brief, frozen (-80o C) stock cultures were streaked out on a blood agar plate (BAP) and left to grow overnight at 37oC and 5% CO2. A single bacterial colony was isolated and suspended in 3 mL of Lysogeny broth/Luria-Bertabi (LB) medium (Becton, Dickinson and Co., Sparks, MD) and agitated overnight at 37ºC and 5% CO_2. Overnight cultures were diluted 1:50 in 50 mL of fresh pre-warmed LB broth in a 250 ml erylenmeryer flask and grown to early/mid-log phase (OD600 = 0.2-0.5). Next, 2 mL of the concentrated culture sample was removed. Cells were washed twice using pre-chilled (4ºC) phosphate buffered saline (PBS), pelleted by centrifugation (5000 rpm for 3 min), then re-suspended in 1 mL of sterile PBS. The bacterial density was estimated via direct count using a Petroff-Hauser Counting Chamber and confirmed by serial dilution and plating on LB agar, and then diluted to the desired cell concentration, 1×10^7 CFU/mL in cold PBS.

Micro-Computed Tomography Analysis: Rats anesthetized with isoflurane (2%) were imaged at 12 weeks post-injury using a SkyScan 1176 in-vivo hi-resolution micro-CT (Bruker-MicroCT, Kontich, Belgium) with the following settings: 89kV polychromatic x-ray beam; current of 256 µA and an exposure time of 81 milliseconds for each of 180 rotational steps. Two independent investigators blinded to cohort assignment reviewed the micro-CT images (170-200 flattened longitudinal m-CT slices/rat) on CT-Analyser (Bruker-MicroCT, Kontic, Belgium) and selected regions of interest (ROI) on every fifth slice encompassing ectopic bone. The binary selected slices were then used to perform 3D image analysis yielding a total volume of HO in the selected area of interest.

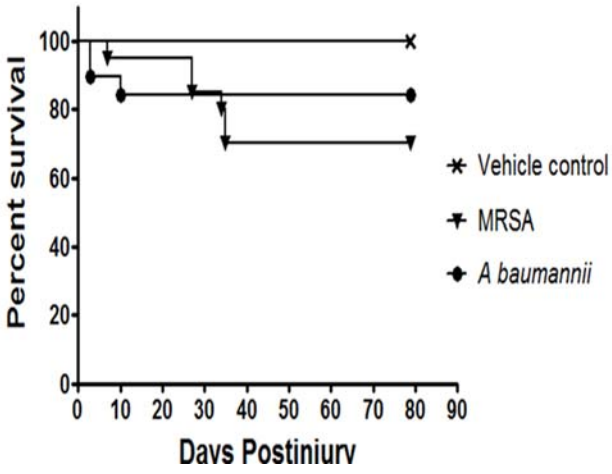


Figure 1. Survival outcomes of injured rats wherein the traumatized muscle surrounding the amputation site at the time of closure was infected with either MRSA (1×10^6) or *A. baumannii* (1×10^6). Kaplan-Meier survival curves are shown. Animals were euthanized if they demonstrated signs of infection after the third débridement of the amputation wound site.

Results-All rats in both experimental groups as well as the control group developed HO. Survival rate among treatment groups was less in the MRSA experimental group (14/20, 70%) in comparison to the Acinetobacter experimental (16/18, 89%) and control (5/5, 100%) groups (Figure 1). Specifically, 6 of 20 rats were euthanized in the MRSA group, chiefly for overwhelming infection persistent after three debridements, which occurred between the 4th and 5th week postoperatively. Whereas two rats inoculated with Acinetobacter were euthanized during week 2 and week 4 for weight loss of greater than 10% of preoperative weight. At 12 weeks, we observed significantly more robust HO on mCT volumetric analysis in animals infected with MRSA (122.32 mm^3 ± 29.22) when compared to Acinetobacter (15.04 mm^3 ± 2.43; $p<0.05$) and controls (11.22 mm^3 ± 2.77; $p<0.05$) (Figure 2). There was no significant difference shown in this measure when comparing Acinetobacter with surgical controls not inoculated with bacteria. Strikingly, the co-infection of wounds with both strains

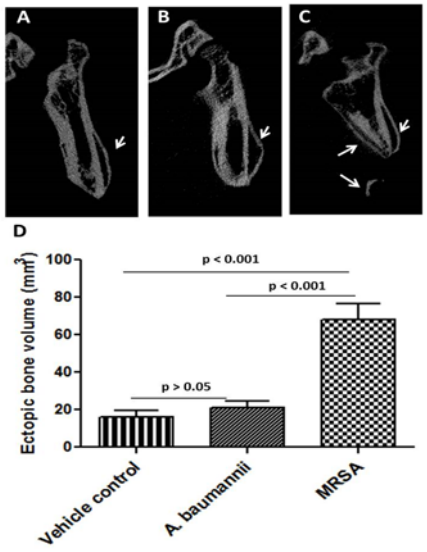


Figure 2. Representative 12 weeks mCT images of rats inoculated with (A) PBS (Non-infected control), (B) A.baumannii and (C) MRSA. (D) Quantification of ectopic bone after 12 weeks post injury.

of bacteria had had no significant effect on HO formation in comparison to uninfected wounds Histologic and bacterial colonization measurements in the soft tissue and in the bone marrow compartment of the residual limb are detailed under the Task 2 accomplishments listed below.

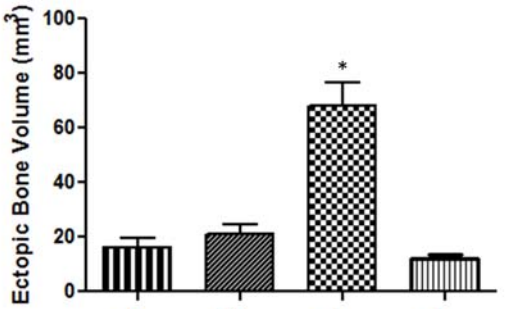


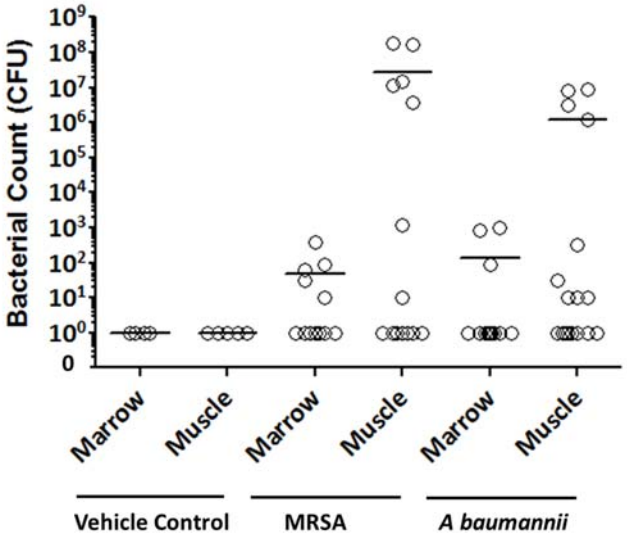
Figure 3. Effect of polymicrobial wound infection on trauma-induced HO formation.

Conclusion/Significance-Our findings suggest that MRSA infection (inoculation of 1 x 10^6 organisms per wound), refractory to antibiotic treatment, results in early development and more pronounced HO formation. The dose of bioburden used is ample to warrant preceding with follow-on characterization studies. The absence or presence of systemic antibiotic treatment has no effect on outcome measures, therefore follow-on experiments will precede in the absence of antibiotic prophylaxis.

Task2: Optimize HO frequency

Using the described model of trauma induced mode HO, we sought to evaluate if the presence of bioburden (*A. baumannii* or MRSA) impacts the magnitude of ectopic bone formation and to determine what effects persistent infection has on the late microbial flora at the amputation site.

Methods: We subjected (48) Sprague-Dawley adult male rats (450-550g) rats to blast overpressure, femur fracture, soft-tissue crush injury at the fracture site and subsequent immediate trans-femoral amputation through the zone of injury. Wounds were inoculated beneath the myodesis with vehicle (n=8) or 1×10^6 CFUs of *A. baumannii* (n=20) or MRSA (n=20). Animals were monitored for evidence of wound drainage or dehiscence and debridement was performed when these clinical manifestations of infection were noted. Animals were euthanized if residual limb wound drainage persisted after three debridements or the animal showed overwhelming symptoms of systemic infection. We performed microCT imaging 12 weeks post-



operatively in order to measure ectopic bone volume. Samples of muscle tissue adjacent to the amputation site and bone marrow from the residual femur were harvested to determine persistence of infection. Representative animals in each treatment group underwent en bloc resection of the femur and surrounding musculature for hematoxylin and eosin staining.

Table1: List of bacteria present in the marrow compartment and soft tissue 12 weeks post injury.

	Vehicle control	*Acinetobacter baumannii*	Methicillin Resistant *Staphylococcus aureus* (MRSA)
Marrow	Negative	*Arcanobacterium haemolyticum; Enterobacter cloacae* and *Enterococcus faecalis*	*Staphylococcus aureus*
Muscle Tissue	Negative	*Arcanobacterium haemolyticum, Streptococcus porcinus, Staphylocucus cohnii ssp urealyticum, Staphylococcus xylosus, Gardnerella vaginalis, Pasteurella multocida, Enterobacter cloacae* and *Enterococcus faecalis*	*Staphylococcus aureus*

Figure 3:Bacterial counts [in colony forming units (CFUs) converted to log scale] in the marrow compartment and soft tissue of rats infected with vehicle control (PBS; noninfected control), MRSA, and *A. baumannii* after 12 weeks. Each data point represents the actual CFU value for each animal in each treatment group while the horizontal bar indicates the mean CFU for each treatment group. All rats inoculated with MRSA tested positive for MRSA, whereas rats inoculated with *A. baumannii* tested positive for other microorganisms as detailed in **Table 1** (above).

Results: Thirty-eight rats survived the duration of our study, of which all formed HO. At 12 weeks, we observed more severe HO in rats infected with MRSA (68.9 mm^3 ± 8.6) when compared to *A. baumannii* (20.9 mm^3 ± 3.7) or vehicle (16.3 mm^3 ± 3.2). Muscle

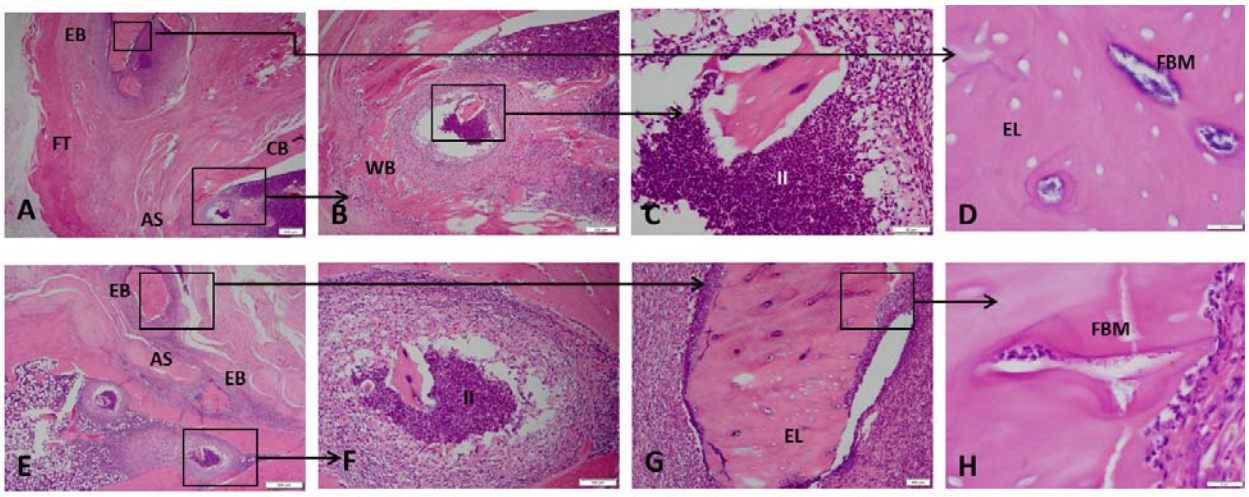


Figure 4. Histology of ectopic bone formation in MRSA-treated rats at 12 weeks is shown in A-H (Stain, Hematoxylin and eosin). For detailed evaluation, images of six selected regions at higher magnification are shown. In the medullary space and soft tissue, there is evidence of chronic inflammation, neutrophil infiltration, purulent infection, osteomyelitis, and necrotic ectopic bone as indicative of empty osteocytic lacunae containing bacterial microcolonies. AS = amputation site; CB = cortical bone; EL = empty lacunae; EB = ectopic bone; FBM = foci of bacterial microcolonies; FT = fibroblastic tissue; II = intramedullary infection; WB = woven bone.

tissue and marrow harvested from rats inoculated with *A. baumannii* tested negative for *A. baumannii* infection but were positive for other strains of bacteria (1×10^2 – 8.6×10^6 CFU), whereas muscle soft-tissue and bone marrow from MRSA-infected rats contained MRSA only (> 1 x 10^5 CFU) (Figure 3 and Table 1). Histologic analysis of residual limbs that were inoculated with MRSA demonstrated evidence of ectopic bone formation in the muscle soft-tissue noted to be necrotic. Furthermore, chronic soft tissue infection and increased neutrophil infiltration can also be appreciated (Figure 4).

Outcome measures evaluating the effects of increased crush injury time from 1 minute to 5 minutes (20.0) were highly variable and inconsistent in augmenting HO development in comparison to a 1 minute crush injury time.

<u>*Conclusion*</u>: Our findings suggest that infection with MRSA but not *A. baumannii* is associated with an increased volume of ectopic bone formation. These findings may be associated with the persistence of MRSA, however additional studies are required to elucidate the mechanism for the differences we observed. Our findings suggest that persistent MRSA infection, refractory to antibiotic treatment, may result in chronic local inflammation leading to increased HO formation. Osteomyelitis or chronic local tissue infection particularly when colonized by MRSA and Acinetobacter strains used for this experiment may be associated with more robust HO formation. The presence of other bacteria in the *Acinetobacter baumannii* inoculated group may indicate co-colonization and is deserving of further study. This work emphasizes the importance of the initial wound debridements in addition to the systemic and perhaps local antimicrobial therapies geared towards decreasing bioburden in combat wounds

Task 3: Evaluate reproducibility of model, morphology and biomarkers

During the this annual performance period, the robustness and reproducibility of the our rat HO model and the effects of bioburden on augment HO formation has been established (findings are summarized in the attached CORR 2015 publication). Additionally, various molecular, histologic, and microbiologic analyses on early HO tissue have been conducted in regard replicate studies detailed and are summarized in Tasks 1 & 2 above. Other studies to assess circulating levels of system inflammatory mediators (cytokines and chemokines) as well as chondrogenic and osteogenic mRNA gene transcripts in early HO tissue are on-going.

KEY RESEARCH ACCOMPLISHMENTS

- Published peer-reviewed manuscript entitled “The development of a rat model to investigate the formation of blast-related post-traumatic heterotopic ossification” which characterizes our rat model of combat-related HO that incorporates the critical elements associated with combat injury, specifically a systemic blast injury, femur fracture with soft tissue crush, and transfemoral amputation through the zone of injury wherein all animals develop radiographic evidence of HO within 2 months post injury.
- Completed experiments testing the in vivo effects of microbial bioburden (*A. baumannii* and MRSA) on HO.
- Completed studies evaluating the effects of polymicrobial infection (MRSA plus A. baumannii) on HO formation.
- Published peer-reviewed manuscript entitled “Effect of Bioburden on Heterotopic Ossification Formation in an Established Rat Model” was accepted for publication in the journal Clinical Orthopaedics and Related Research.
- We submitted a new IACUC protocol to continue the work scope covered in this grant. Specifically, experiments to evaluate the effects of increasing the soft-tissue compression (crush injury) from 1 minute to 5 minutes on ectopic bone formation.
- Completed studies assessing the impact of crush injury time (1 minute versus 5 minute) on HO development. Findings suggest that additional crush injury time had no significant impact on HO development in non-MRSA infected wounds.
- Proteomic and gene expression biomarker studies focused on assessing local/systemic inflammation as well as early osteogenic signaling within the zone of injury are currently on-going and are expected to be completed by Q5.

CONCLUSION

Our experimental findings suggests that of the two most common bacterial isolates of combat-related amputations, MRSA infection results in the development of a several fold increase in the volume of ectopic bone compared with A. baumannii and a vehicle control in a rat model. Persistent infection with MRSA results in a greater volume of ectopic bone formation, which may be the result of chronic soft tissue inflammation, and that early wound colonization may be a key risk factor. Interventions that mitigate wound contamination and inflammation (such as early débridement, systemic and local antibiotics) may also have a beneficial effect with regard to the mitigation of HO formation, and should be evaluated with that potential in mind in future preclinical studies.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Lay Press

Nothing to report

Peer-Reviewed Scientific Journals

1. Polfer EM, Hope DN, Elster EA, Qureshi AT, Davis TA, Golden D, Potter BK, Forsberg JA. The development of a rat model to investigate the formation of blast-related post-traumatic heterotopic ossification. Bone Joint J. 2015 Apr;97-B(4):572-6. doi: 10.1302/0301-620X.97B4.34866.

2. Pavey GJ, Qureshi AT, Hope DN, Pavlicek RL, Potter BK, Forsberg JA, Davis TA. Bioburden Increases Heterotopic Ossification Formation in an Established Rat Model. Clin Orthop Relat Res. 2015 Mar 31. [Epub ahead of print]. PMID:25822455

Invited Articles

Nothing to report

Abstracts

Gabriel J. Pavey, Ammar T. Qureshi, Donald N. Hope, Rebecca L. Pavlicek, Benjamin K. Potter, Thomas A. Davis, Jonathan A. Forsberg. Evaluation of Bioburden on the Development of Heterotopic Ossification in an Established Rat Model. Aug 18-21 2015. MHSRS. Ft. Lauderdale, FL

Presentations

Donald N. Hope, Dana M. Golden, Ammar Qureshi, Kyle Brunette, Elizabeth M. Polfer, Benjamin K. Potter, Thomas Davis, Jonathan A. Forsberg. Evaluation of Early Heterotopic Ossification and the Effect of Bioburden in an Established Rat Model. August 18-21, 2014. MHSRS, Ft Lauderdale, FL

Gabriel J. Pavey, Ammar T. Qureshi, Donald N. Hope, Rebecca L. Pavlicek, Benjamin K. Potter, Thomas A. Davis, Jonathan A. Forsberg. Evaluation of Bioburden on the Development of Heterotopic Ossification in an Established Rat Model. March 7, 2015. Maryland Orthopaedic Association. Baltimore, MD

Gabriel J. Pavey, Ammar T. Qureshi, Donald N. Hope, Rebecca L. Pavlicek, Benjamin K. Potter, Thomas A. Davis, Jonathan A. Forsberg. Evaluation of Bioburden on the Development of Heterotopic Ossification in an Established Rat Model. March 28-30, 2015. Orthopedic Research Society. Las Vegas, NV.

Gabriel J. Pavey, Ammar T. Qureshi, Donald N. Hope, Rebecca L. Pavlicek, Benjamin K. Potter, Thomas A. Davis, Jonathan A. Forsberg. Evaluation of Bioburden on the Development of Heterotopic Ossification in an Established Rat Model. July 15-18, 2015. Southern Orthopaedic Association. Asheville, NC

INVENTIONS, PATENTS AND LICENSES

Nothing to report

REPORTABLE OUTCOMES

Nothing to report

OTHER ACHIEVEMENTS

Nothing to report

REFERENCES

Nothing to report

APPENDICES

PDFs of the peer-reviewed publication listed below stemming from this research proposal are attached.

1. Quad Chart
2. Polfer EM, Hope DN, Elster EA, Qureshi AT, Davis TA, Golden D, Potter BK, Forsberg JA.The development of a rat model to investigate the formation of blast-related post-traumatic heterotopic ossification. Bone Joint J. 2015 Apr;97-B(4):572-6. doi: 10.1302/0301-620X.97B4.34866.
3. Pavey GJ, Qureshi AT, Hope DN, Pavlicek RL, Potter BK, Forsberg JA, Davis TA. Bioburden Increases Heterotopic Ossification Formation in an Established Rat Model. Clin Orthop Relat Res. 2015 Mar 31. [Epub ahead of print]. PMID:25822455

Combat-Related Heterotopic Ossification: Development of Animal Models for Identifying Mechanisms & Testing Therapeutics

Log # 10083004

W81XWH-14-2-0010

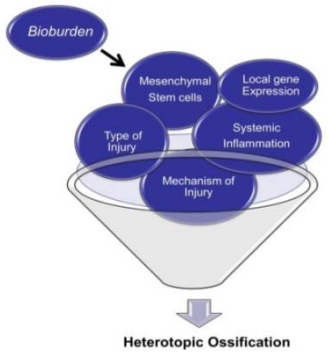
PI: CDR Forsberg **Org:** Naval Medical Research Center **Award Amount:** $347,947

Study/Product Aim(s)

- Determine the effects of *Acinetobacter baumannii* and *Methicillin Resistant Staphylococcus Aureus* (MRSA) on the development of heterotopic ossification in a following blast-related severe fracture and amputation in rat model of traumatic injury.
- Evaluate the effects of uni-microbial versus mixed infection.
- Determine for the presence of persistent tissue and marrow infection
- Characterize local and systemic inflammatory responses post-injury.
- Examine potential HO biomarkers using multianalytic proteomic and gene expression assays.

Approach

We will use a rat model of combat-related HO that incorporates the critical elements associated with combat-injury specifically a blast injury, a fracture-crush, transfemoral amputation through the zone of injury followed by inoculation of the wound site with A. baumannii and/or MRSA. We will monitor and characterize the early onset development of HO histological and radiogaphically using mCT analysis . Activation of osteogenic development in injured soft tissues will be examined by histology, gene expression and proteomic technologies.



Accomplishments: Results from this research were published in two peer-reviewed orthopaedic journals. We have fully characterized our rat model of blast-related traumatic injury, described the effect of bioburden inoculation on ectopic bone formation, and assessed the impact of extended crush injury time on traumatic extremity injury and ectopic bone development.

Timeline and Cost

Activities CY	14	15		
Amend IACUC protocol				
Uni-microbial infection studies				
Mixed infection studies				
Histologic, gene expression, bacteriology and proteomic analysis				
Estimated Budget ($348k)	**$61k**	**$287k**	**$000**	**$000**

Updated: (July 15, 2015)

Goals/Milestones

CY15 Goal – IACUC approval, pilot infection studies and radiographic, histologic, proteomic and gene expression analyses.

- ☐ IACUC approval completed
- ☐ Pilot infection studies completed
- ☐ Radiographic analysis and volumetric analysis ectopic bone formation of pilot infection studies completed
- ☐ Effects of polymicrobial infection studies completed
- ☐ Manuscripts published in BJJ and CORR
- ☐ Evaluation of crush injury time completed
- ☐ Molecular, proteomic and histological characterization of "best model" (in progress)

Comments/Challenges/Issues/Concerns: None

Budget Expenditure to Date

Cumulative expended : $207,142.52

NCE approved until December, 2015

■ GENERAL ORTHOPAEDICS

The development of a rat model to investigate the formation of blast-related post-traumatic heterotopic ossification

E. M. Polfer,
D. N. Hope,
E. A. Elster,
A. T. Qureshi,
T. A. Davis,
D. Golden,
B. K. Potter,
J. A. Forsberg

From Naval Medical Research Center, Maryland, United States

Currently, there is no animal model in which to evaluate the underlying physiological processes leading to the heterotopic ossification (HO) which forms in most combat-related and blast wounds. We sought to reproduce the ossification that forms under these circumstances in a rat by emulating patterns of injury seen in patients with severe injuries resulting from blasts. We investigated whether exposure to blast overpressure increased the prevalence of HO after transfemoral amputation performed within the zone of injury. We exposed rats to a blast overpressure alone (BOP-CTL), crush injury and femoral fracture followed by amputation through the zone of injury (AMP-CTL) or a combination of these (BOP-AMP). The presence of HO was evaluated using radiographs, micro-CT and histology. HO developed in none of nine BOP-CTL, six of nine AMP-CTL, and in all 20 BOP-AMP rats. Exposure to blast overpressure increased the prevalence of HO.

This model may thus be used to elucidate cellular and molecular pathways of HO, the effect of varying intensities of blast overpressure, and to evaluate new means of prophylaxis and treatment of heterotopic ossification.

Cite this article: *Bone Joint J* 2015;97-B:572–6

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Bone Joint J 2015;97-B:572–6.
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Heterotopic ossification (HO) is the formation of mature lamellar bone in non-osseous tissues. It usually occurs following trauma and is most common after elbow and acetabular fractures, burns, traumatic brain injury, and spinal cord injury, as well as hip arthroplasty.[1-5] During the last decade, HO has emerged as a prevalent and difficult problem for patients with blast- and other combat-related orthopaedic injuries. Studies performed at military treatment facilities of the United States showed a prevalence of 63% to 65%,[6-8] which is far higher than that reported following civilian trauma. Matsumoto et al[9] reported an incidence of HO in civilian amputations of 22.8%

Limbs that develop HO are prone to several untoward outcomes with an adverse effect on function and quality of life. In addition to pain, HO can affect prosthesis wear in amputees, limit range of movement of joints, ulcerate, or envelop neurovascular structures causing progressive deficits.[10-14] As a result, up to 41% of patients with HO will ultimately require operative excision, a procedure that delays rehabilitation and is fraught with complications.[8] Given the prevalence of HO in combat casualties and the morbidity associated with excision, much research is being directed towards understanding the underlying cellular mechanisms, in order to develop and test various means of prophylaxis and treatment.

Current means of prophylaxis including non-steroidal anti-inflammatory medications, external beam radiotherapy or a combination of these developed for use in the civilian setting.[15-18] However, these options are not feasible or contraindicated in the setting of combat- and blast-related wounds and new methods of prophylaxis are needed. While several animal models exist for the investigation of HO, most are non-physiological and involve the implantation of exogenous proteins and/or cells and do not take into account the systemic and local effects of blast exposure.[19,20] Others do not produce relevant patterns of injury[21-24] that incorporate the critical elements associated with combat-injury specifically a blast injury, femoral fracture, crush and transfemoral amputation through the zone of injury as observed in human combat casualties. Still others result in unacceptably high mortality and lack a controlled systemic injury which can be graded using a reproducible classification scheme.[25] The aim of this study was to reproduce combat-related HO in a rat model by emulating unique, reproducible, patterns of injury similar to those in patients with blast injuries.

Materials and Methods

We used 41 pathogen-free adult male Sprague Dawley rats (Rattus norvegicus; Taconic Farms, Germantown, New York; 400 g to 510 g) that were housed in clean plastic cages on a 12-hour light/dark cycle with access to food (standard chow) and water ad libitum. They were allowed to acclimatise for one week prior to experimentation. They were randomly divided into three groups: (1) blast Overpressure BOP-CTL (sham surgery, n = 10), (2) AMP-CTL (extremity trauma + amputation, n = 10) and (3) BOP-AMP (BOP + extremity trauma + amputation, n = 21). Additional animals were treated according to the BOP-AMP protocol in order to assess the formation of HO at early time points (days 1, 7, 10 and 14 after injury) at the histological level (n = 12).

The rats were anaesthetised with isoflurane (1% to 3%). BOP was delivered to the entirety of the rat by a pneumatically driven shock tube (ORA Inc., Fredericksburg, Virginia) at 120kPa +/- 7kPa, an established method resulting in a pronounced systemic inflammatory response with 70% to 90% survivability.[26-28]

The rats were anaesthetised with isoflurane and received buprenorphine (0.05mg/kg) delivered via intraperitoneal injection. BOP-AMP rats were treated immediately after exposure to the BOP. A drop weight apparatus (University of Alabama, Birmingham, Alabama) was used to create a comminuted femoral fracture in a similar fashion to that previously described.[29] The weight was dropped from a height of 88 cm, which reliably reproduced a comminuted midshaft femoral fracture. Immediately afterwards, a soft-tissue crush injury was created by rotating the fracture site between the two anvils of the support stage. A pressure of 20 pounds per square inch of was applied for one minute as determined using a Chatillon force measurement pressure sensor (AMETEK Inc., Largo, Florida).

Immediately following the femoral fracture and crush injury, the extensor mechanism and hamstrings were released distally through a circumferential incision around the distal femur. Amputation was undertaken through the fracture, in an effort to simulate an amputation performed within the zone of injury. This is a common practice when treating blast wounds, in which the zone of injury is extensive.[30] Devitalised bone was debrided and haemostasis achieved. A myoplasty was performed by suturing the distal hamstring and quadriceps fascia over the residual femur, and the wound was closed with 3-0 polyglactin 910 and 4-0 poliglecaprone 25 sutures (Ethicon Inc., Somerville, New Jersey).

Post-operatively, all rats received sustained release buprenorphine (1.2 mg/kg subcutaneously; Zoopharm, Windsor, Colorado) with repeat dosing after three days. They were monitored twice a day for pain management and wound healing. Clinical studies[31,32] have shown that heightened and prolonged local inflammation at the site of injury affects the formation of HO and the potential cellular and molecular mechanisms involved, therefore none of the animals received non-steroidal anti-inflammatory medications.

The rats were anaesthetised with isoflurane (1% to 3%) prior to radiological analysis. Radiographs were taken weekly for three weeks, then monthly until six months after injury using Orex PcCR 1417 (Carestream Health, Rochester, New York) and a SkyScan 1176 *in vivo* hi-resolution micro-CT (Bruker-MicroCT, Kontich, Belgium). Two orthopaedic surgeons (JAF, BKP) blinded to the study groups reviewed the images to determine the presence or absence of HO.

The rats were killed six months after the injury with Fatal Plus (390 mg/kg i.p.; Patterson Veterinary, Devens, Massachusetts) which contains sodium pentobarbital as the active ingredient. The injured extremity including the residual femur and attached muscles was collected, fixed in 10% formalin, decalcified in 5% formic acid, paraffin-embedded and cut into 5-um longitudinal sections on a microtome for staining (Haematoxylin and Eosin; Histoserv, Inc., Germantown, Maryland).

Statistical analysis. Data were analysed using JMP 9 statistical software (SAS Institute Inc., Cary, North Carolina). We used the Fisher's Exact Test to compare proportions of HO between groups. We defined statistical significance as a two-tailed α of < 0.05, and data are represented as mean ± standard error of the mean (SEM) unless otherwise specified.

Results

A total of 41 rats were randomised into the three groups: 10 BOP-CTL, 10 AMP-CTL, and 21 BOP-AMP. Three rats were killed on the first post-injury day for unrelieved stress and bowel obstruction, probably due to the proximity of the abdomen to the femur in the rat. The 38 remaining rats were included for statistical analysis.

The first radiological signs of HO were observed between two and four weeks post-operatively. In general, HO was apparent earlier in the BOP-AMP group. The prevalence of HO varied between groups ($p < 0.001$). There was no evidence of HO in the BOP-CTL group without concomitant local injury. HO was present in six of the nine AMP-CTL and in all 20 remaining rats in the BOP-AMP group (Fig. 1). Thus, the addition of 120KPa of BOP resulted in a significantly higher prevalence of HO, when comparing the AMP-CTL and BOP-AMP groups ($p = 0.007$) (Fig. 2).

Histological examination showed evidence of chondrocyte hypertrophy, cartilage vascularisation and early mineralisation of the cartilage in BOP + AMP injured rats by 14 days after injury (Fig. 3). Histological assessment six months after the injury showed minimal periosteal new bone formation in BOP-CTL rats (Fig. 4a). In contrast, those subjected to BOP showed hypertrophic zones of disorganised chondrogenesis within the soft-tissues and evidence of endochondral ossification (Figs 4b and 4c).

Discussion

The prevalence of HO in combat casualties is higher than that seen following civilian trauma.[1,2,6,7] Efforts to identify

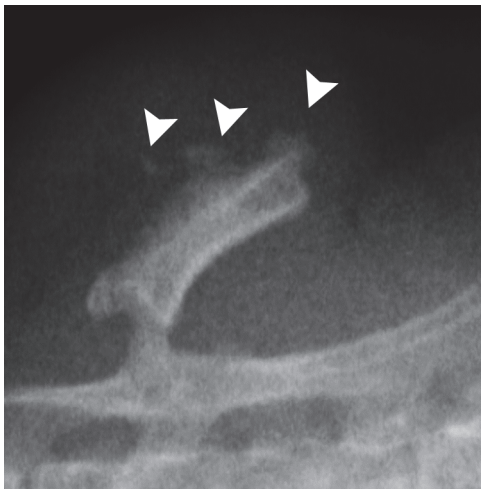

Fig. 1

Anteroposterior radiograph showing heterotopic ossification (arrowheads) in a rat thigh (BOP AMP group) four weeks after injury.

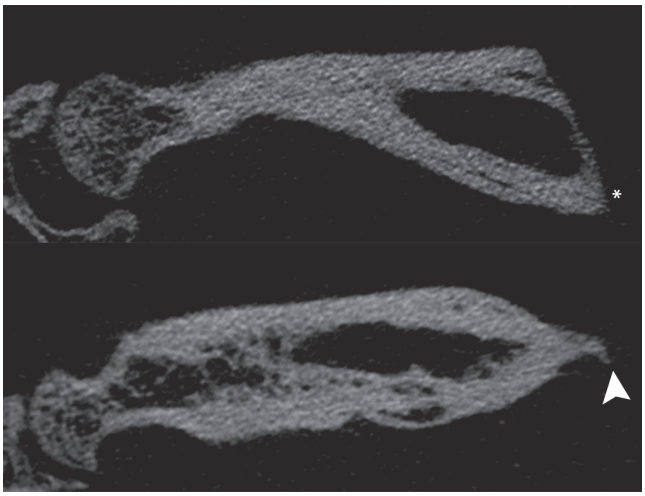

Fig. 2

Micro-CT images showing heterotopic ossification in a rat thigh (BOP-AMP group) eight weeks after injury.

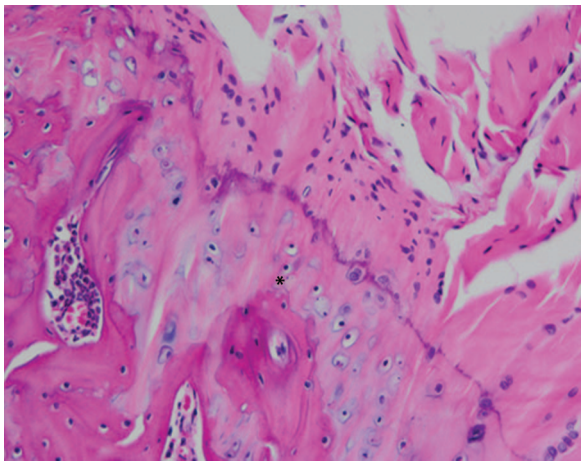

Fig. 3

Haematoxylin and eosin staining performed on muscle tissue adjacent to the amputation site (lower left) 14 days after injury. Note chondrocyte hypertrophy, cartilage vascularisation and mineralisation of the cartilage (asterisk) within the injured muscle tissue (top right). (x 100 magnification)

new means of prophylaxis in blast wounds depend on the development of a suitable and reproducible animal model for preclinical testing. There are promising molecules in development for which preclinical testing also requires an appropriate animal model.[32-34] Here, we demonstrate that HO developed in all 20 rats exposed to BOP, thigh crush, femoral fracture, and transfemoral amputation through the zone of injury. Concomitant exposure to BOP increased the prevalence of HO compared with those in the AMP-CTL group. These results suggest that blast injury when combined with severe trauma is likely to be an important component to the development of HO.

Exposure to BOP increased the prevalence of HO in this model. Although not yet investigated in this model, possible causes for this include the release of inflammatory neuropeptides. For example, substance-P, acting through the neurokinin-1 receptor (NK1), and calcitonin gene-related peptide (CGRP) result in recruitment, accumulation and degranulation of mast cells leading to remodelling and release of osteogenic precursors from nerve.[35] Likewise, mice treated with NK1 antagonists and mast cell knockout show little or no HO formation in models that incorporate exogenous BMP.[36]

Several small animal models of HO have been described,[37] although few recreate the injury pattern observed in combat casualties. The most common model involves the implantation of a bone morphogenetic protein (BMP)-containing matrix as first described by Urist.[38] A variation of this involves the intramuscular implantation of Matrigel containing BMPs, which allows for the relatively sustained release of BMP-2 and/or BMP-4 at the implantation site within an extracellular matrix environment.[23,39] Other models that induce local trauma include a hip arthroplasty model,[22] the Michelsson/Immobilisation-Manipulation model[21] the Achilles tenotomy model,[20] and blunt force models.[23,24] Unfortunately, none of these reproduce the pattern of injury which incorporates the clinical risk factors associated with combat-injury, specifically a blast injury with a femoral crush-fracture and transfemoral amputation through the zone of injury wherein all animals develop radiological evidence of HO within two months of injury.

A rat model that resembles combat injuries has been described.[25] However, it is unlikely to gain favour within the research community. In this model, the authors created a traumatic amputation using a high explosive. However, the portion of the rat proximal to the knee (or elbow) was shielded from the blast wave, ostensibly limiting the animal's total body exposure to the BOP. The authors also did not quantify the degree and consistency of the BOP, nor the

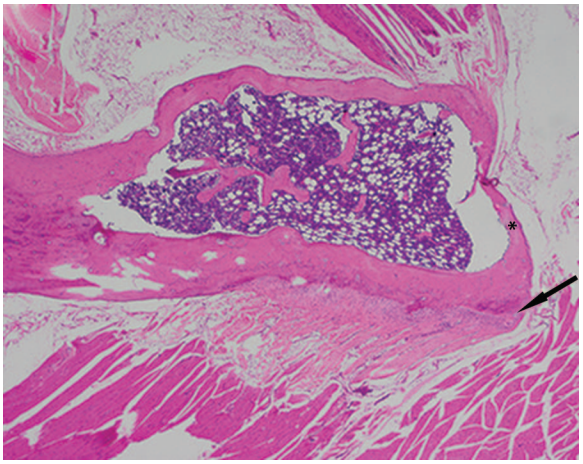

Fig. 4a

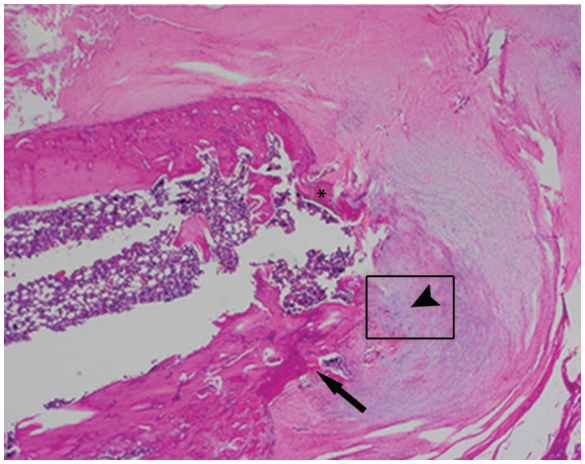

Fig. 4b

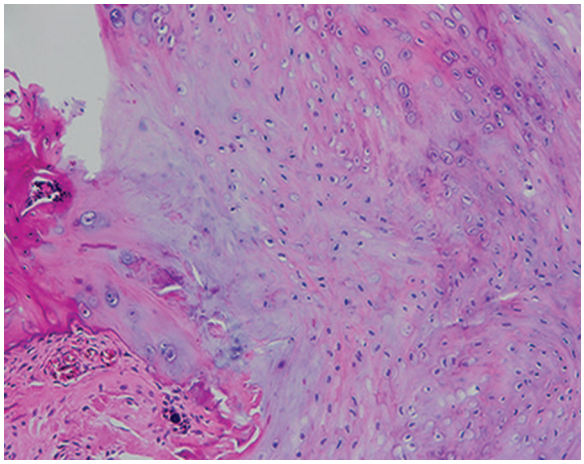

Fig. 4c

Longitudinal sections of residual femora (Haematoxylin & Eosin). a) AMP-CTL group, note the residual femur (asterisk), minimal periosteal new bone formation (arrow) but no evidence of endochondral ossification (x 40 magnification). b) BOP-AMP group with abundant periosteal new bone formation (arrow) and copious endochondral ossification (arrowhead) (x 40). c) A higher magnification view of the area outlined. Note the immature osteoid (pink) and the cartilage matrix (blue) (x 100).

energy delivered to the animal, which we believe may contribute to variation in the local and/or systemic inflammatory response. In addition, the use of a high explosive requires a facility which cannot be reproduced in most research facilities, and it is not likely to be translatable to larger animal models for various sociopolitical reasons.[40] However, by emulating unique patterns of injury seen in patients with severe injuries using a highly controlled and well-described series of interventions, as we have done, we believe the proposed model is likely to be more reproducible and more widely accepted.

This study has limitations inherent in those conducted on one small animal species. It is possible that a large animal model may more closely mimic the underlying physiological processes seen in combat casualties by allowing the incorporation of other variables such as serial debridement procedures and negative pressure wound therapy which is difficult in a small animal model. We also did not attempt to test individual components of the composite extremity injury pattern (e.g., fracture, amputation, or crush injury) alone to discern their relative contributions to the formation of HO. However, by standardising the injury as we have done, these experiments are not only possible, but better suited to elucidate the effect of BOP on the formation of HO. Finally, we have not yet assessed the impact of bioburden in the form of bacterial or fungal contamination, or ischaemia that occurs after injury or after the use of a tourniquet, or varying degrees of BOP, which may also affect the prevalence and/or extent of HO. We did not fully characterise the early development of HO in the development of this model. However, future experiments designed to evaluate the early cellular signaling and gene expression patterns in addition to assessing prophylactic treatments are in progress.

In summary, we successfully modelled blast-related HO in a rat by recreating a combat- injury pattern using a series of precise, reproducible interventions. The BOP-AMP group demonstrated HO in all surviving animals with acceptable mortality. Future studies may use this model to investigate

early cellular and molecular pathways, test the effects of various intensities of BOP, and evaluate new means of prophylaxis and treatment currently in development.

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SYMPOSIUM: RESEARCH ADVANCES AFTER A DECADE OF WAR

Bioburden Increases Heterotopic Ossification Formation in an Established Rat Model

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Abstract

Background Heterotopic ossification (HO) develops in a majority of combat-related amputations wherein early bacterial colonization has been considered a potential early risk factor. Our group has recently developed a small animal model of trauma-induced HO that incorporates many of the multifaceted injury patterns of combat trauma in the absence of bacterial contamination and subsequent wound colonization.

Questions/purposes We sought to determine if (1) the presence of bioburden (*Acinetobacter baumannii* and methicillin-resistant *Staphylococcus aureus* [MRSA]) increases the magnitude of ectopic bone formation in traumatized muscle after amputation; and (2) what persistent effects bacterial contamination has on late microbial flora within the amputation site.

Methods Using a blast-related HO model, we exposed 48 rats to blast overpressure, femur fracture, crush injury, and subsequent immediate transfemoral amputation through the zone of injury. Control injured rats (n = 8) were inoculated beneath the myodesis with phosphate-buffered saline not containing bacteria (vehicle) and treatment rats were inoculated with 1×10^6 colony-forming units of *A baumannii* (n = 20) or MRSA (n = 20). All animals formed HO. Heterotopic ossification was determined by quantitative volumetric measurements of ectopic bone at 12-weeks postinjury using micro-CT and qualitative histomorphometry for assessment of new bone formation in the residual limb. Bone marrow and muscle tissue biopsies

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Each author certifies that his or her institution approved the animal protocol for this investigation and that all investigations were conducted in conformity with ethical principles of research.
This work was performed at the Naval Medical Research Center, Silver Spring, MD, USA.

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were collected from the residual limb at 12 weeks to quantitatively measure the bioburden load and to qualitatively determine the species-level identification of the bacterial flora.

Results At 12 weeks, we observed a greater volume of HO in rats infected with MRSA (68.9 ± 8.6 mm^3; 95% confidence interval [CI], 50.52–85.55) when compared with *A baumannii* (20.9 ± 3.7 mm^3; 95% CI, 13.61–28.14; $p < 0.001$) or vehicle (16.3 ± 3.2 mm^3; 95% CI, 10.06–22.47; $p < 0.001$). Soft tissue and marrow from the residual limb of rats inoculated with *A baumannii* tested negative for *A baumannii* infection but were positive for other strains of bacteria ($1.33 \times 10^2 \pm 0.89 \times 10^2$; 95% CI, -0.42×10^2–3.08×10^2 and $1.25 \times 10^6 \pm 0.69 \times 10^6$; 95% CI, -0.13×10^6–2.60×10^6 colony-forming units in bone marrow and muscle tissue, respectively), whereas tissue from MRSA-infected rats contained MRSA only ($4.84 \times 10^1 \pm 3.22 \times 10^1$; 95% CI, -1.47×10^1–11.1×10^1 and $2.80 \times 10^7 \pm 1.73 \times 10^7$; 95% CI, -0.60×10^7–6.20×10^7 in bone marrow and muscle tissue, respectively).

Conclusions Our findings demonstrate that persistent infection with MRSA results in a greater volume of ectopic bone formation, which may be the result of chronic soft tissue inflammation, and that early wound colonization may be a key risk factor.

Clinical Relevance Interventions that mitigate wound contamination and inflammation (such as early débridement, systemic and local antibiotics) may also have a beneficial effect with regard to the mitigation of HO formation and should be evaluated with that potential in mind in future preclinical studies.

Introduction

Blast injuries present formidable surgical, treatment, and rehabilitation challenges. The resulting wounds are multifaceted, often resulting in composite tissue loss, comminuted open fractures, and frequent traumatic amputations. Related wound contamination is ubiquitous, often with multidrug-resistant organisms such as *Acinetobacter baumannii* and methicillin-resistant *Staphylococcus aureus* (MRSA), often calling for protracted treatment regimens that include serial surgical débridements and broad-spectrum antibiotic therapy [2, 3, 6]. A survey of wound infections from Combat Support Hospitals in Iraq from 2003 to 2004 demonstrated a relatively high frequency of MRSA (26%) followed by *Acinetobacter calcoaceticus-baumannii* complex (11%), *Klebsiella pneumoniae* (13%), and *Pseudomonas aeruginosa* (10%) in combat-related injuries [6]. Wound infection-related complications include wound dehiscence, deep soft tissue infection, biofilm development on orthopaedic implants, and infectious osteomyelitis, often leading to chronic, debilitating infections, further bone and soft tissue destruction, and subsequent limb amputation [2, 20, 22, 23, 33].

Heterotopic ossification (HO) is the formation of mature lamellar bone within soft tissue after severe traumatic injury [10]. It is known to develop in the majority of combat-related amputations, and early bacterial colonization has been considered a potential early risk factor [12, 13]. However, the cellular and early signaling mechanism(s) for combat injury-induced HO formation remain unclear. Recent findings suggest that the heightened and prolonged expression of inflammatory and other reparative mediators may be contribute to HO formation [11, 14]. Moreover, the combat wound appears to provide a unique microenvironment conducive to osteogenesis that promotes the skewed differentiation of endogenous tissue-derived progenitor cells toward ectopic bone development within injured and healing soft tissue [10].

We previously developed a rat model of combat-related HO that incorporates the critical elements associated with combat injury, specifically a systemic blast injury, femur fracture with soft tissue crush, and transfemoral amputation through the zone of injury wherein all animals develop radiographic evidence of HO within 2 months postinjury [25]. Expanding on this model, in this study, we sought to evaluate if (1) the presence of bioburden (*A baumannii* and MRSA) increases the magnitude of ectopic bone formation in traumatized muscle after amputation; and (2) what persistent effects bacterial contamination has on late microbial flora within the amputation site.

Materials and Methods

Animals

Forty-eight young adult pathogen-free male Sprague-Dawley rats (*Rattus norvegicus*; 12–14 weeks, 400–500 g) were purchased from Taconic Farms (Germantown, NY, USA). All animals were housed in clean plastic cages and kept on a 12-hour light/dark cycle with unlimited access to food (standard rodent chow) and fresh water ad libitum. The study protocol (12-OUMD-20s) was reviewed and approved by the Walter Reed Army Institute of Research/Naval Medical Research Center Institutional Animal Care and Use Committee in compliance with all applicable Federal regulations governing the protection of animals in research.

Bacteria Culture Conditions

The *A baumannii* (strain 5075) and MRSA (MRSA strain 107261) organisms used in this study are highly virulent, well-characterized clinical specimens isolated from combat wounds from patients treated at the Walter Reed National Military Medical Center. In brief, frozen (−80 °C) stock cultures were streaked out on a blood agar plate and left to grow overnight at 37 °C and 5% CO_2. A single bacterial colony was isolated and suspended in 3 mL of Lysogeny broth/Luria-Bertabi medium (Becton, Dickinson and Co, Sparks, MD, USA) and agitated overnight at 37 °C and 5% CO_2. Overnight cultures were diluted 1:50 in 50 mL of fresh prewarmed Luria-Bertabi broth in a 250-mL Erlenmeyer flask and grown to early/midlog phase (OD_{600} = 0.2–0.5) where cells proliferate in a logarithmic fashion under optimal culture and nutrient conditions resulting in a controlled cell growth rate. Next, 2 mL of the concentrated culture sample was removed. Cells were washed twice using prechilled (4°C) phosphate-buffered saline (PBS), pelleted by centrifugation (5000 rpm for 3 minutes), then resuspended in 1 mL of sterile PBS. The bacterial density was estimated through direct count using a Petroff-Hauser Counting Chamber (Hauser Scientific, Horsham, PA, USA) and confirmed by serial dilution and plating on Luria-Bertabi agar and then diluted to the desired cell concentration, 1×10^7 colony-forming units (CFU)/mL in cold PBS.

Rat Model of Trauma-induced HO and Bacterial Inoculation

A total of 48 rats were exposed to blast overpressure exposure, femur fracture, soft tissue crush injury, and limb amputation as previously described [25]. After quadriceps myoplasty, three muscle sites immediately surrounding the amputation site were inoculated with: (1) vehicle (100 µL of PBS; n = 8); (2) *A baumannii* (100 µL of 1×10^7 CFU; n = 20); or (3) MRSA (100 µL of 1×10^7 CFU; n = 20). Closure of the incision was performed using a 3-0 Vicryl in the deep subcutaneous tissue and a running 4-0 subcuticular Monocryl. Wounds were covered in Vetbond (3M Animal Care Products, St Paul, MN, USA). Postoperatively rats were monitored at least twice daily by animal care staff, research investigators, and veterinarians for animal activity, signs of pain, weight loss, wound dehiscence, or infectious tracts for the duration of the study. Wounds that exhibited signs of infection defined as drainage, progressive marginal erythema, or dehiscence were débrided. Rats were euthanized if they demonstrated signs of infection after a third débridement. We conducted a power analysis based on the effect of a projected 50% increase in ectopic bone volume within the soft tissue surrounding the amputated femur when the injury site was inoculated with either MRSA or *A baumannii*. Using conservative assumptions and data from our prior studies, the power analysis showed that with eight rats per treatment group and $\alpha = 0.05$, there is 90% power to detect a 50% increase in ectopic bone volume. Thus, it was anticipated that 20 rats in the infected treatment groups would provide adequate statistical power to detect treatment effects of moderate size on the major outcome variable of ectopic bone volume even with attrition of as many as 12 rats per group. All eight rats in the control group survived until the 12-week micro-CT scan (Fig. 1). Six animals in the MRSA group were euthanized during the fourth and fifth weeks for overwhelming infection. Two of the rats in the *A baumannii* group died on the day of surgery and were excluded. A low level of mortality after surgery was consistent with findings during model development and represents the devastation of these multifaceted injuries, particularly given that blast overpressure of 120 ± 7 kPa itself is calibrated for 70% to 90% survivability [1, 7, 25]. In addition, two rats infected with *A baumannii* were euthanized for sustained weight loss greater than 10% during postoperative weeks 2 and 4.

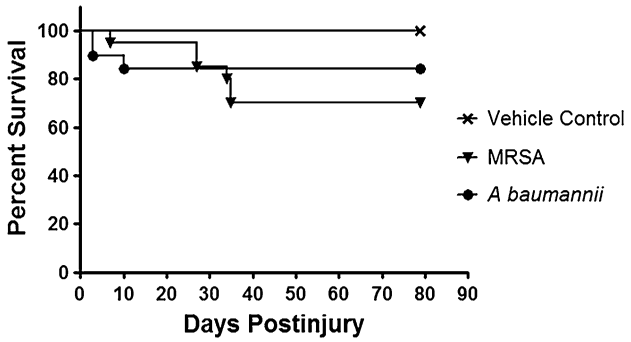

Fig. 1 Treatment effects on survival outcome of injured rats wherein the traumatized muscle surrounding the amputation site at the time of closure was infected with either MRSA (1×10^6) or *A baumannii* (1×10^6). Kaplan-Meier survival curves are shown. Animals were euthanized if they demonstrated signs of infection after the third débridement and irrigation of the amputation wound site.

Micro-CT Analysis

Rats anesthetized with isoflurane (2%) were imaged at 12 weeks postinjury using a SkyScan 1176 in vivo high-resolution micro-CT (Bruker-MicroCT, Kontich, Belgium) with the following settings: 89-kV polychromatic xray beam, current of 256 µA, and an exposure time of 81 msec for each of 180 rotational steps. Two investigators (GJP, ATQ) independently reviewed the micro-CT images (170–200 flattened longitudinal micro-CT slices/rat) on a CT-

Analyser (Bruker-MicroCT) and calculated the volume of ectopic bone formation using selected regions of interest on every fifth slice encompassing ectopic bone. The binary selected slices were then used to perform three-dimensional image analysis yielding a total volume of HO in the selected area of interest.

Sample Collection and Culture

After the micro-CT scans were assessed for image quality and clarity, scanning efficiency, and reconstructed for volumetric analysis of ectopic bone formation, rats were euthanized with pentobarbital (Fatal Plus; 390 mg/kg intraperitoneally; Patterson Veterinary, Devens, MA, USA). Muscle tissue adjacent to the amputation site and femur was aseptically excised. Femurs were removed and separated from the soft tissues. Bone marrow from the residual femur was extruded from the medullary canal by flushing using a 10-mL syringe fitted with an 18-gauge needle with 10 mL of sterile PBS after proximal and distal osteotomies. Samples were diluted in PBS out to 10^{-6}, plated on a blood agar plate, and incubated overnight at 37° C, 5% CO_2. Colonies were counted and screened for differing morphology. Isolates were streaked on a blood agar plate for direct bacterial species identification using the BD Phoenix automated microbiology system in accordance with the manufacturer's instructions (BD Diagnostics, Sparks, MD, USA).

Histological Analysis

At the time of euthanasia, two rats from each treatment group received an en bloc resection of the residual limb, which was then fixed in 10% formalin, decalcified in 5% formic acid, paraffin-embedded, cut into 5-μm longitudinal sections on a microtome, and stained using hematoxylin and eosin stain (Histoserv, Inc, Germantown, MD, USA). Histologic tissue samples were qualitatively analyzed for evidence of soft tissue cartilage formation, inflammation, lamellar bone formation within the soft tissues, the presence of persistent inflammatory cells, or active bacterial infection. The histopathological analysis was conducted by a veterinary pathologist (CH) blinded to the treatment groups.

Statistical Analysis

Kaplan-Meier modeling was performed to assess the survivability patterns of the control and treatment groups over the duration of the study. Intraclass correlation coefficient (ICC) was calculated to assess the reliability of interobserver measurements of HO formation using the micro-CT analyzing software. Analysis of variance modeling was used to determine whether there was a significant difference in the volume (mm^3) of ectopic bone measured among the three groups followed by the Tukey's honestly significant difference test to determine the mean difference among the three groups. All data, including the bacterial CFU counts, were presented as mean ± SD with 95% confidence interval (CI) unless specified otherwise. Exact p values were stated except when < 0.001. All statistical analysis described previously was performed using the RStudio, Version 0.98.953 (© 2009–2013 RStudio Inc, Boston, MA, USA).

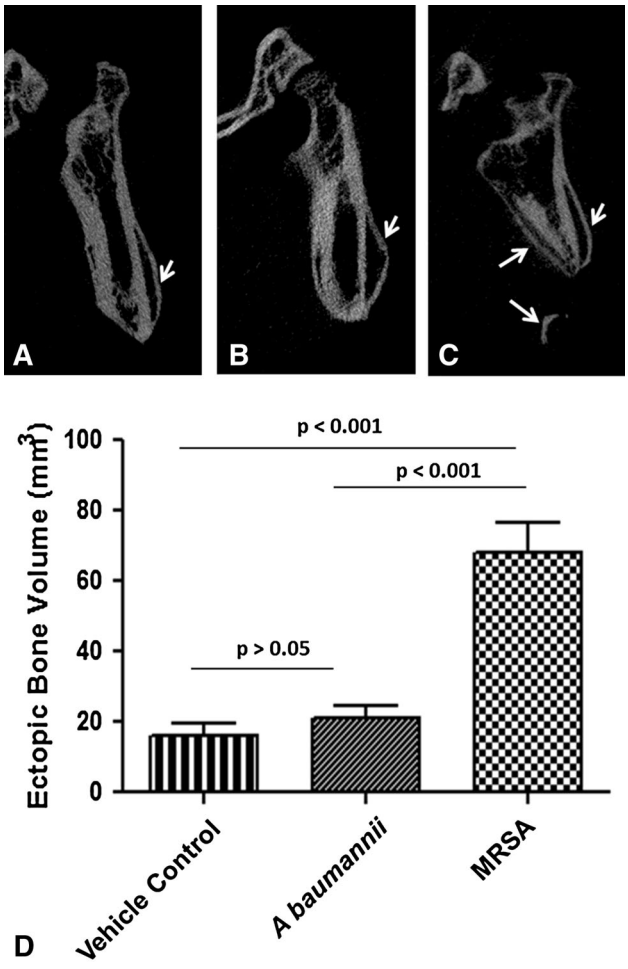

Fig. 2A–D MRSA infection increases trauma-induced ectopic bone formation. Representative longitudinal 12-week micro-CT images of the residual femurs of rats inoculated with (**A**) vehicle control (PBS; noninfected control); (**B**) *A baumannii*; and (**C**) MRSA are shown. The white arrows highlight the areas of ectopic bone formation. (**D**) The amount of ectopic bone was quantified 12 weeks postinjury from vehicle control (n = 8), *A baumannii* (n = 16), and MRSA (n = 14) treatment groups. Result expressed are expressed as the mean ± SD.

Results

All rats that survived to the end of the study (*A baumannii* [n = 16], MRSA [n = 14], vehicle [n = 8]) demonstrated the formation of ectopic bone on the 12-week micro-CT scan (Fig. 2A–C). Volumetric measurements of ectopic bone formation (Fig. 2D) were more robust in animals inoculated with MRSA (68.0 ± 8.6 mm^3; 95% CI, 50.52–85.55) than *A baumannii* (20.9 ± 3.7 mm^3; 95% CI, 13.61–28.14; $p < 0.001$) and vehicle control (16.3 ± 3.2 mm^3; 95% CI, 10.06–22.47; $p < 0.001$). Comparison of vehicle control and *A baumannii*-inoculated groups showed no difference ($p = 0.43$) with excellent interobserver agreement (ICC = 0.98).

Soft tissue and bone marrow collected from the residual femur from rats inoculated with *A baumannii* tested negative for *A baumannii* flora but were positive for other strains of bacteria ($1.33 \times 10^2 \pm 0.89 \times 10^2$; 95% CI, -0.42×10^2–3.08×10^2 and $1.24 \times 10^6 \pm 0.69 \times 10^6$; 95% CI, -0.13×10^6–2.60×10^6 CFU in bone marrow and muscle tissue, respectively), whereas tissue from MRSA-infected rats contained MRSA only ($4.84 \times 10^1 \pm 3.22 \times 10^1$; 95% CI, -1.47×10^1–11.1×10^2 and $2.80 \times 10^7 \pm 1.73 \times 10^7$; 95% CI, -0.60×10^7–6.20×10^7) (Table 1). Specifically, bacterial culture results from the surviving MRSA-infected rats showed that in eight of 14 rats the muscle tissue surrounding the amputation site was positive for persistent MRSA infection, whereas five of the 12 bone marrow samples that were available after en bloc resection of two animals were MRSA-positive (Fig. 3). En bloc resection performed on MRSA rats demonstrated evidence of bacterial microcolonies, increased neutrophil infiltration, chronic soft tissue infection, and osteomyelitis (foci of bacterial microcolonies, purulent intramedullary infection, and evidence of bone necrosis indicative of empty osteocytic lacunae with islands of necrotic endochondral bone throughout the skeletal muscle; Fig. 4). All rats inoculated with *A baumannii* tested negative for the inoculated bacteria in both the soft tissue and bone marrow cultures. However, nine of 16 soft tissue samples and three of the 14 available bone marrow samples had positive cultures at 12 weeks with various bacterial flora species (Fig. 3). En bloc resection of *A baumannii* residual limbs sent for histology showed inflammatory cells indicative of chronic infection (data not shown); however, representative tissue sections (Fig. 4) failed to capture the periosteal reaction and ectopic bone formation observed on micro-CT (Fig. 2). No bacterial CFUs were detectable in the tissue cultures from vehicle-treated rats.

Discussion

Blast injuries are devastating to the extremities and often include comminuted open fractures, neurovascular injury, soft tissue loss, and traumatic amputations. Wounds often are contaminated with foreign material and microorganisms, including *A baumannii* and MRSA, which have been proven to exhibit multidrug resistance or relatively high virulence, respectively [2, 6, 16, 21]. Because HO develops within the residual limbs of most blast- and otherwise combat-related amputations [3, 12, 13, 26], considerable efforts are directed toward treating symptoms conservatively; however, surgical resection is ultimately necessary

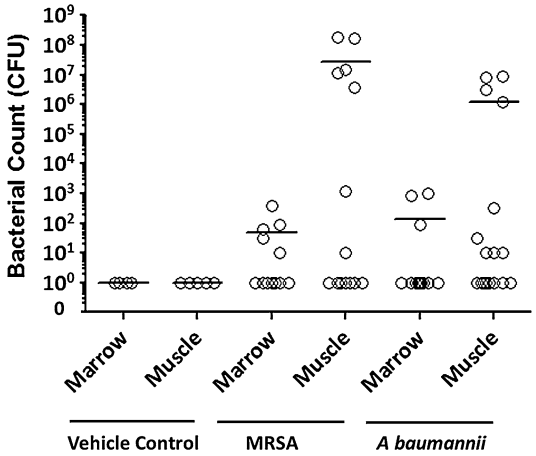

Fig. 3 Bacterial titers (in CFUs converted to log scale) in the marrow compartment and soft tissue of rats infected with vehicle control (PBS; noninfected control), MRSA, and *A baumanni* after 12 weeks are shown. Each data point represents the actual CFU value for each animal in each treatment group, whereas the horizontal bar indicates the mean CFU for each treatment group. All rats inoculated with MRSA tested positive for MRSA, whereas rats inoculated with *A baumannii* tested positive for other microorganisms as detailed in Table 1.

Table 1. List of bacteria present in the marrow compartment and soft tissue 12 weeks postinjury

Tissue	Vehicle control	*Acinetobacter baumannii*	Methicillin-resistant *Staphylococcus aureus*
Marrow	Negative	*Arcanobacterium haemolyticum*; *Enterobacter cloacae* and *Enterococcus faecalis*	*S aureus*
Muscle	Negative	*Arcanobacterium haemolyticum, Streptococcus porcinus, Staphylococcus cohnii ssp urealyticum, Staphylococcus xylosus, Gardnerella vaginalis, Pasteurella multocida, Enterobacter cloacae,* and *Enterococcus faecalis*	*S aureus*

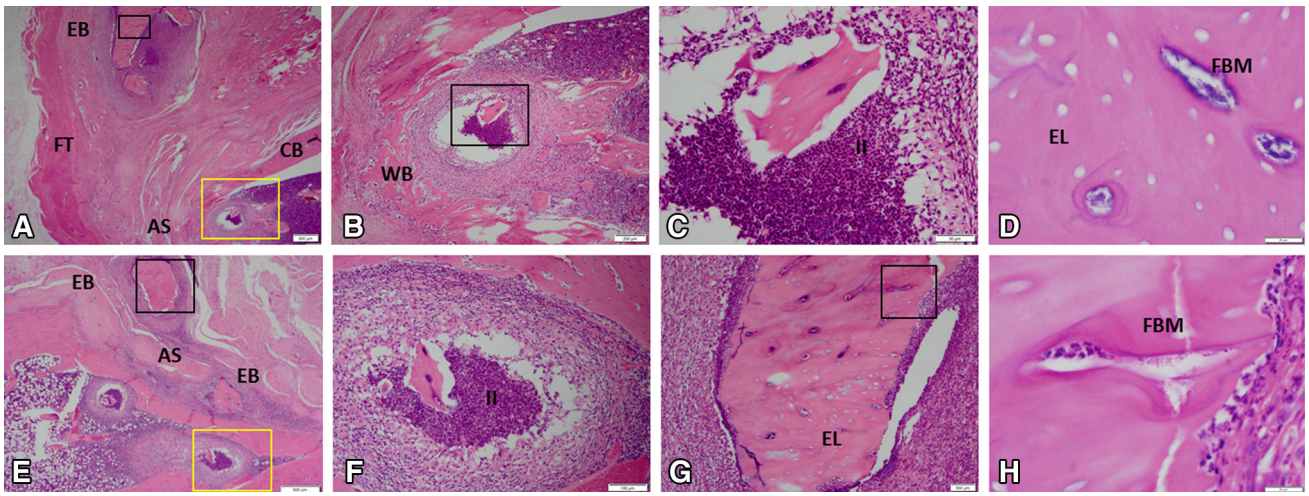

Fig. 4A–H The histological features of ectopic bone formation in MRSA-treated rats at 12 weeks are shown in **A**–**H** (**A**, Stain, hematoxylin and eosin; **A** original magnification, × 1.25; **B**, original magnification, × 4, yellow boxed region in **A**; **C**, original magnification, × 20, black boxed region in **B**; **D**, original magnification, × 100, black boxed region in **A**; **E**, original magnification, × 2; **F**, original magnification, × 10, yellow boxed region in **E**; **G**, original magnification, × 10, black boxed region in **E**; **H**, original magnification, × 10, black boxed region in **G**). For detailed evaluation, images of six selected regions at higher magnification are shown. In the medullary space and soft tissue, there is evidence of chronic inflammation, neutrophil infiltration, purulent infection, osteomyelitis, and necrotic ectopic bone as indicative of empty osteocytic lacunae containing bacterial microcolonies. AS = amputation site; CB = cortical bone; EL = empty lacunae; EB = ectopic bone; FBM = foci of bacterial microcolonies; FT = fibroblastic tissue; II = intramedullary infection; WB = woven bone.

in up to 41% of amputees with HO [30]. As such, considerable focus has been directed toward prevention and mitigation of HO formation; however, understanding factors that exacerbate its development is an important prerequisite. In this effort, we explored, using an established blast-related HO animal model [25], the impact of *A baumannii* or MRSA colonization on the volume of HO formation and identified the characteristics of chronic infection in each setting.

There are several limitations to our study. First, the rat model is not conducive to many of the surgical modalities used in the treatment of traumatic wounds such as serial débridements with negative pressure wound therapy, which are implicated as putative contributors to HO formation [12]. Second, most war wounds are typically colonized by polymicrobial flora [6]. As such, an inoculum of a specific bacterial pathogen (1×10^6 CFU) does not fully address the synergistic role that polymicrobial infection may have in the persistence and virulence of infection plus it limits our ability to assess differences in HO formation or persistence of infection with varied degrees of infection. Preliminary experiments demonstrated that the bacterial concentration of MRSA used in these studies resulted in established persistent infections with high reproducibility and minimal variation in regard to wound complications. Moreover, it has been reported that approximately 50% of combat wounds become clinically infected ($> 1 \times 10^5$ CFU) as opposed to merely contaminated [2, 28]. Notably, as a limitation in identifying the presence of all persistent microorganisms, only aerobic wound microflora were cultured from soft tissue and bone marrow. With the expressed intent of describing the impact of microbial bioburden on trauma-induced HO, we acknowledge our limited description of other forms of HO such as genetic and neurogenic. Neurogenic HO has been well described in civilian populations [8, 15], whereas the focus in military research has been predominantly in traumatic HO. In the neurogenic form, neurotransmitters such as glutamate, substance P, and catecholamines act to induce osteoblasts to form ectopic bone within a permissive local environment [5, 18, 27]. Therefore, the induction of progenitor cells with varying osteoinductive factors is common in both traumatic and neurogenic; however, the difference lies in the elevated levels of systemic and local inflammatory cytokines in the former and neurotransmitters in the latter [14]. That having been said, the expression and/or production of inflammatory mediators in this current study was not assessed; therefore, inferences regarding the role of local infection on HO development are based only on histopathological changes noted at study termination.

Contamination of residual limbs with MRSA, but not *A baumannii,* contributed to the volume of HO that developed in this rat model. This finding is relevant given that MRSA is the predominant organism in 35% to 50% of clinically infected combat wounds [3, 4]. Often, these wounds demand serial débridements to achieve healthy-appearing and/

or culture-negative tissue. Despite this, approximately one-fourth of amputations develop late infection after closure of a healthy-appearing wound bed [30]. More débridements, five to seven to be exact, are associated with the development of HO, likely resulting from mechanical trauma to the tissue as well as the systemic inflammatory responses that can result from repeated returns to the operating room [7, 19]. In comparison, our MRSA-inoculated rats developed a greater volume of HO with most (11 of 14 analyzed) doing so in the absence of the described serial surgical interventions, further suggesting MRSA involvement. An unexpected finding of our study is the relative lack of effect of *A baumannii* on ectopic bone formation despite the selection of the strain based on its clinical ubiquity and relative virulence [2, 29]. This result may be expected given that *A baumannii*-infected rats cleared their infection. Another explanation involves the signaling of toll-like receptors (TLRs), which are found to be expressed on osteogenic precursor cells [24]. Interestingly, purified lipopolysaccharide, a ligand found on Gram-negative organisms, which has preferential affinity for toll-like receptor 4 (TLR4), demonstrated slow activation of mesenchymal stem cells; however, prolonged exposure to the toxin resulted in decreased expression of TLRs. Alternatively, downregulation of TLRs did not occur with prolonged exposure to the Gram-positive specific cell wall component lipoteichoic acid, perhaps allowing for osteogenic differentiation of mesenchymal stem cells by Gram-positive organisms like MRSA [19, 31]. It may be that infection with Gram-negatives such as *A baumannii* affects HO development indirectly in clinical practice. This organism is not found in wounds at the time of injury but rather is a nosocomial pathogen found in combat theater hospitals. Infection of wounds during aeromedical evacuation or at combat hospitals may "reinfect" wounds, resulting in serial débridements and negative pressure wound therapy, factors that some infer may contribute to HO development [6, 12, 23].

Chronic infection, like persistently symptomatic HO, can delay or regress the rehabilitation of blast- and otherwise war-related amputees. After combat-related lower extremity amputations, 27% require return to the operating room for wound infection [30] at some point during their hospitalization. In a study of 110 service members with severe orthopaedic wounds that subsequently developed osteomyelitis, a retrospective review showed that *A baumannii* accounted for 70% of initial admissions to the hospital; however, these responded well to treatment, making up only 6% of recurrences. By comparison, MRSA, presented as the infecting organism in only 8% of initial diagnoses of osteomyelitis, however, was responsible for 31% of readmissions for osteomyelitis with Gram-positives as a whole accounting for 75% of recurrence [33]. In addition, modeling of war wounds in a rabbit demonstrated that monobacterial Gram-negative inoculation at a titer of 1×10^5 *A baumannii* failed to produce osteomyelitis, whereas polymicrobial inoculum and/or those containing MRSA demonstrated active, persistent, infection 8 weeks postinoculation [32]. Our findings also support the persistence of Gram-positive infections with 56% and 21% rates of soft tissue contamination and osteomyelitis in our MRSA cohort, the latter occurring despite the bone not being directly inoculated. Conversely, *A baumannii* was not present in any 12-week cultures, but rather other antibiotic-resistant ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus, K pneumoniae, A baumanii, P aeruginosa*, and *Enterobacter* species) such as enteric organisms *Enterococcus faecium* and *Enterobacter cloacae* as well as various Gram-positive staphylococci, but not *S aureus* were isolated at time of culture (Table 1). Negative culture results for *A baumannii* infection 12 weeks postinoculation are consistent with previous rat studies and may be the result of decreased virulence in bone as compared with other infection sites [9]. Secondary infection with other nosocomial pathogens, particularly Gram-positive organisms, is consistent with clinical findings and suggests that initial infection with *A baumannii* may produce an environment conducive to secondary infection or overgrowth of other nosocomial organisms [17]. Although the synergism between initial *A baumannii* infection and subsequent infection still needs to be studied, this result may be informative for clinical treatment plans.

Our study suggests that of the two most common bacterial isolates of combat-related amputations, MRSA infection results in the development of a several-fold increase in the volume of ectopic bone compared with *A baumannii* and a vehicle control in a rat model. This difference may be related to the microorganisms' persistent colonization and invocation of chronic infection because this difference was shown in our study minus the surgical treatment already known to influence HO formation. Therefore, in addition to drug therapies that target signaling pathways in bone development and/or proinflammatory osteogenic mediators, we further propose that initiation of prophylactic local and/or systemic Gram-positive antimicrobial therapy at the time of injury and continued treatment of subclinical infection may help mitigate the formation of ectopic bone; further preclinical work, to include assessment of polymicrobial infections, impact of differential TLR signaling, and the evaluation of systemic and/or local antimicrobial interventions, is necessary to further elucidate this effect.

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